PHASE SEPARATION OF MISCIBLE PHOSPHOLIPIDS BY SONICATION OF BILAYER VESICLES

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ABSTRACT Sonication of phospholipid vesicles may result, according to their liquid or solid crystal state, in the generation of unilamellar vesicles or structural defects within their bilayers, respectively. The transition temperature T_m of the phospholipid bilayer is usually the threshold temperature delineating the physical effects of ultrasound. However, for vesicles made from a mixture of two miscible phospholipids, this threshold temperature was not found to be the intermediate T_m of the phospholipid mixture bilayers, but the T_m of the lowest melting component. This was due to a simultaneous lateral phase separation of the two phospholipids induced by the sonication as demonstrated by differential scanning calorimetry analysis.

INTRODUCTION

Studies on the physical effects of ultrasound on multilayered phospholipid vesicles have shown that they closely depend on the temperature of the vesicle suspension during sonication. At a temperature above the transition temperature (T_m) of the phospholipid bilayers, single-wall vesicles are progressively generated (Papahadjopoulos and Miller, 1967). At a temperature below this T_m, short sonication bursts induce structural defects within the bilayers that result in high ion permeation (Lawaczeck et al., 1975; 1976). These observations have allowed us to carry out a procedure to separate physically the aqueous compartment from the lipidic lamellae: after sonication of multilamellar vesicles and ultracentrifugation, the aqueous compartment, and lipidic lamellae could be recovered in the supernatant and in the pellet, respectively (Bakouche and Gerlier, 1983), provided that the sonciation was done below a threshold temperature. In contrast, after sonication above this threshold temperature, phospholipids and aqueous phase were recovered in both the supernatant (as unsedimented small vesicles) and in the pellet. According to Lawaczek et al. (1975, 1976), this threshold temperature should be the temperature at which the phospholipids would change from solid to liquid crystal state, namely the T_m of the bilayers, and this was verified with vesicles made from a pure phospholipid. It is reported here that it is not the case for vesicles made from an ideal mixture of two phospholipids since ultrasound could induce simultaneously a lateral phase separation of the two miscible phospholipids.

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MATERIALS AND METHODS

Chemicals and Solutions

Dipalmitoyl-D-L-α-phosphatidylcholine (DPPC); dimyristoyl-L-α-phosphatidylcholine (DMPC); and distearoyl-L-α-phosphatidylcholine (DSPC) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C-DPPC, di[1-^{14}C] palmitoyl-L-α-phosphatidylcholine, and [^{14}C]-DSPC, di[1-^{14}C] stearoyl-(L-α-phosphatidylcholine were obtained from Amersham International plc (Buckinghamshire, England). 5,6 Carboxylfuorescein (5,6 CF, Eastman Kodak, Rochester, NY) was further purified according to Ralston et al. (1981) by absorption onto activated charcoal, followed by chromatography onto a LH-20 Sephadex column (Pharmacia, Uppsala, Sweden) to eliminate hydrophobic impurities.

Preparation of Multilamellar Vesicles

As previously described (Gerlier et al., 1983), a homogeneous film of either DMPC or DPPC or DMPC and DPPC or DPPC and DSPC in equimolar ratio was made by drying the lipid or the lipid mixture from chloroform/methanol solutions (2 vol/1 vol). The film was dispersed by intermittent stirring and heating, at a temperature 2 K above the T_m of the highest melting component, for 2 min, either in 5,6 CF 30 mM solution or in distilled water. After four washes in either Tris-HCl buffer (0.05 M, pH 7.5) or distilled water for DSC analysis, and centrifugation at 48,000 × g for 30 min, the vesicles were finally suspended in the appropriate buffer at 6–7 mg of lipids per milliliter.

Sonication Procedure and Separation of Aqueous Compartment from Lipidic Lamellae

The liposomes were submitted to six cycles of probe sonication alternating 30-s burst (20 KHz, 25 W) and 30-s rest, followed by ultracentrifugation (Bakouche and Gerlier, 1983). The temperatures during the bursts (burst temperature) and during the rests (rest temperature) were maintained by a water bath to keep variations of the temperature of the vesicle suspension due to the probe heating within less than ±1 K. After equilibrium at 277 K, 100 μl of the suspension were ultracentrifuged at 132,000 *g* for 5 min in an Airfuge ultracentrifuge (Beckman, Palo Alto, CA). The supernatant was kept for subsequent analysis and the pellet was

washed three times before being resuspended in 100 μl. 5,6 CF was measured by use of a spectrofluorimeter with 490-nm excitation and 520-nm emission wavelengths after addition of 1% Nonidet P40 (Fluka AG, Buchs, Switzerland) and appropriate dilution. [^{14}C]DPPC and [^{14}C]DSPC were determined by liquid scintillation counting. Phospholipids were determined according to a modified Barklett procedure (Gerlier et al., 1983).

Differential Scanning Calorimetry

After sonication, the vesicles (made in the presence of distilled water) were cooled down to 277 K, pelleted and resuspended at 21.8 mg ml^{-1} phospholipids in cold distilled water. The vesicle suspension (31–36.7 mg) was transferred into an aluminum crucible that was immediately sealed. DSC measurements were carried out by means of a heat flow DSC Mettler apparatus (model TA2000B; Mettler Instrumente AG, Zurich, Switzerland). Calibrations for temperature and heat measurement were made using the melting point and the enthalpy of melting of high purity metals. A precision of ±0.2 K was obtained. Argon gas flow in the apparatus was chosen on the ground of its density and its low thermal conductivity. Experiments were done in the temperature range 278–318 K. The calorimeter was set at a constant temperature (278 K) and, when the signal was constant, the temperature was increased with a heating rate of 0.015 K s^{-1}. The calorimetric signal was read by an HP3455A digital voltmeter controlled by a HP85 computer. Data were stored and used off line. The sampling period was 5 s.

RESULTS AND DISCUSSION

Vesicles made from pure DPPC and containing self-quenched 5,6 CF were submitted to sonication at 312 K or 316 K, i.e., just above or just below the DPPC T_m (314.5 K), and further ultracentrifuged. When sonication was performed beneath the DPPC T_m a complete separation of aqueous compartment and lipidic lamellae was observed (Table I). Above the T_m, the sonication of the vesicles did not result in an efficient compartment separation. Therefore, the threshold temperature delineating the ability of the ultrasound to make the liposomes fully leaky required that the phospholipid be in a gel crystal state, as previously observed for the induction of structural defects (Lawaczeck et al., 1975, 1976). These structural effects were stable since the release of 5,6 CF was almost complete and of greater magnitude than one that would have resulted from a transient diffusion outside the bilayers (Bakouche and Gerlier, 1983).

Liposomes made from an ideal mixture in equimolar ratio of either [^{14}C]-DPPC and DSPC or DPPC and [^{14}C]-DSPC were sonicated at a few degrees below or above the T_m of each phospholipid. A complete separation of the aqueous probe 5,6 CF from the phospholipid could be obtained after ultracentrifugation only when sonication was performed at a temperature below the 314.5 K, the T_m of the lowest melting component (DPPC, Table I). Whereas after sonciation at a temperature above the T_m of the highest melting component (328 K, DSPC) both phospholipids were recovered in the supernatant, after sonication at temperatures between the two T_m's, the floating phospholipids were solely the lowest melting component DPPC (Table I). Similar results were obtained with liposomes made from the ideal mixture of DMPC and DPPC (data not shown). From these data, it could be concluded that toward the generation of structural defects or unilamellar vesicles by ultrasounds, the behavior of each phospholipid was independent of each other and was related to its crystal state delineated by its own T_m.

Such results would have been expected if the two phospholipids would have been nonintersoluble and reputed to melt independently (Mabrey and Sturtevant, 1976; Op den Kamp et al., 1975; Arnold et al., 1981): the T_m of the lowest melting component as the threshold temperature would then have been in agreement with the requirement for each phospholipid to be in a solid crystal state to allow the generation of structural defects. But, DPPC/DSPC (Shimshick and Mc Connell, 1973; Phillips et al., 1970) and DMPC/DPPC (Mabrey-Gaud, 1981) mixed bilayers behave as an ideal mixture and the phospholipids are considered to melt simultaneously at an intermediate temperature between their own T_m's, which delineate their actual physical state. The DPPC/DSPC and the DMPC/DPPC complexes are considered to be in a

TABLE I
DISTRIBUTION OF PHOSPHOLIPIDS AND 5.6 CF WITHIN SUPERNATANT AND PELLET AFTER SONICATION OF MULTILAMELLAR LIPOSOMES MADE FROM ONE OR TWO PHOSPHOLIPIDS IN EQUIMOLAR RATIO: T_m OF THE LOWEST MELTING COMPONENT AS UPPER THRESHOLD TEMPERATURE FOR THE GENERATION OF STRUTURAL DEFECTS

Phospholipid composition	Liposome compartment	Temperature during sonication 312°K			316°K			323°K			331°K		
		5,6CF	Phospholipid	dpm	5,6CF	Phospholipid	dpm	5,6CF	Phospholipid	dpm	5,6CF	Phospholipid	dpm
DPPC	Supernatant	99.6	<1		68.2	31.6							
	pellet	0.014	97		30.6	67.3							
[^{14}C]DPPC and DSPC	Supernatant	99.6	<1	0.025	77.2	13	26.4	78.6	15	27.2	57.5	36	33.2
	pellet	0.009	97	96.6	19.1	86.3	68.7	18.5	84.1	68.8	40.5	64	67.5
DPPC and [^{14}C]DSPC	Supernatant	98.2	<1	0.069	75.6	10.4	0.07	79	14	0.08	56.2	31	42.5
	pellet	0.013	96	94.6	17	89	94.3	20.1	86.2	93.1	43.6	68.8	57.2

Results were expressed as a percentage of material initially present within the liposomes, 5,6CF being measured after addition of 1% NonidetP40.

gel crystal state below their intermediate T_m, 319.5 and 304.5 K, respectively (Shimshick and Mc Connell, 1973; Phillips et al., 1970; Mabrey-Gaud, 1981). Therefore, for the generation of structural defects the threshold temperature should be the intermediate T_m, and not the T_m of the lowest melting component as observed for DMPC/DPPC and DPPC/DSPC mixture (Table I and not shown). To explain this discrepancy, and from the observation on the generation of small vesicles composed from only one initial phospholipid component under certain conditions (see Table I), it was hypothesized that sonication may also induce a lateral phase separation between the two miscible components resulting in a situation similar to that of the nonideal mixture.

Vesicles made from a mixture of DMPC and DPPC in equimolar ratio in distilled water and sonicated at 293, 302, or 306 K were cooled back to 277 K, pelleted and analyzed by DSC. As shown on Fig. 1 *a*, unsonicated DMPC/DPPC bilayers behaved as an ideal mixture since only one main T_m (or intermediate T_m) was observed ~304.5 K, as previously reported (Mabrey-Gaud, 1981). After sonication at 293 K, below the T_m of the lowest melting component (296 K, DMPC), a similar DSC curve was observed (Fig. 1 *b*). By contrast, after sonication at 302 or 306 K, between the DMPC and DPPC T_m's and below or above the intermediate DMPC/DPPC T_m, two main transition temperatures were observed ~296 K and 314.5 K, corresponding to the DMPC and DPPC T_m's, respectively (Fig. 1 *c*). These DSC curves were very similar

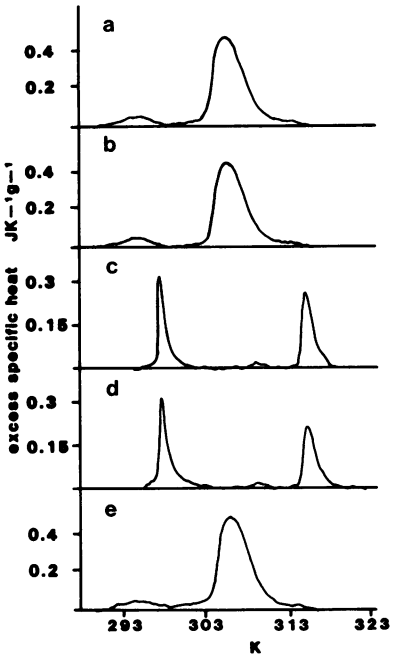

FIGURE 1 DSC curves showing the phase transitions of (*a*) unsonicated DMPC/DPPC vesicles, (*b*) DMPC/DPPC vesicles sonicated at 293 K, (*c*) DMPC/DPPC vesicles sonicated at 302 K or 306 K, (*d*) a mixture of unsonicated DMPC vesicles and DPPC vesicles, and (*e*) DMPC/DPPC vesicles sonicated at 302 K and further incubated at 318 K.

to that of a mixture of vesicles made from either pure DMPC or pure DPPC (Fig. 1 *d*). In addition, the calorimetric scans did not show the marked changes in the transition endotherm that are characteristic of unilamellar small vesicles generated by ultrasound (Mabrey-Gaud, 1981). This confirmed that the sedimentable phospholipids obtained after only short bursts of sonication were unlikely to be small vesicles. Since, under sonication at temperature between the DMPC and DPPC T_m's, small vesicles composed solely from the lowest melting component were generated and recovered in the supernatant after ultracentrifugation (see Table I), it could be questioned whether in the phospholipid bilayer pellets (the only ones analyzed by DSC) a parting of the two types of phospholipids into different bilayers has also occurred. However, such a parting was unlikely to occur: the warming of the sonicated DMPC/DPPC vesicles suspension for a few minutes at 318 K, that is above the T_m of the highest melting component, before the DSC analysis or the scanning from high to low temperature of the same sample allowed the mixed phospholipid bilayers to recover a thermal diagram similar to that of unsonicated DMPC/DPPC vesicles with a unique main T_m ~304.5 K (Fig. 2 *e* and unshown data). Since ~25% of the lowest melting component has been lost as unsedimented vesicles (see Table I), the DMPC/DPPC molar ratio of the sonicated phospholipid pellet was shifted from equimolar to 4.3/5.7 molar ratio. As previously reported (Mabrey-Gaud, 1981), such a change in the molar ratio should have shifted the T_m to a slightly higher value (~305 K), but no attempt was made to evidence this T_m shift with a sufficient precision. This last result indicates that an homogeneous redistribution of DMPC and DPPC molecules has occurred during the incubation of the sonicated vesicles at a temperature at which both phospholipids were in liquid crystal state. This redistribution was probably not the result of heterogeneous vesicle fusion since this could not be observed when heating a mixture of vesicles made either from DMPC or DPPC. This observation clearly favors that both DMPC and DPPC were present in every bilayer of the sedimented DMPC/DPPC vesicles obtained after their sonication at a temperature between the DMPC and DPPC T_m's. This was confirmed by recent data obtained from experiments based on the strange property of α-lactalbumin to disrupt phospholipid vesicles at pH 4 only when incubated at a temperature close to the phospholipid bilayer T_m (Hanssens et al., 1983). Sedimented DMPC/DPPC vesicles obtained after sonication at a temperature intermediate between DMPC T_m and DPPC T_m could be fully disrupted after their incubation with α-lactalbumin at a temperature close to the DMPC T_m or to the DPPC T_m (data not shown).

In conclusion, besides the two known effects of ultrasound on phospholipid bilayers, induction of structural defects when they are in gel crystal state (Lawaczeck et al., 1975) and generation of unilamellar vesicles when they are in liquid crystal state (Papahadjopoulos and Miller, 1967),

ultrasound may also induce a lateral phase separation between two miscible phospholipids when applied at a temperature comprised between their individual T_m's. This lateral phase separation does not require for the mixed bilayers to be in a gel or liquid state (i.e., occurs below and above the intermediate T_m), occurs within 30 s of sonication (data not shown), and remains stable provided that the phospholipid bilayer is not warmed above the T_m of the highest melting component. Ultrasonic propagation gives rise to periodic changes of local pressure and temperature (Mitaku and Date, 1982) and it can be questioned whether the lateral phase separation results from a difference in the behavior of the two miscible phospholipids toward these local physical changes. It should be stressed that a change in pressure has been reported in several models to modify the thermal phase diagram: for example, an increase in pressure of an homogeneous binary mixture in liquid state can induce the selective solidification of one of the components (see for review Pistorius, 1976). Therefore, it is not unlikely that the phase separation induced by sonication within a certain range of temperatures could be explained by a similar modification of the thermal phase diagram of the two component lipid mixture under local pressure. Finally, the possible interference of ultrasounds on the intermolecular relationship within biological membranes could bring a new insight in the biological effect of ultrasounds.

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